REMARKS

Applicants request acceptance of the enclosed paper copy and computer readable form (floppy disk) of the Sequence Listing. The sequence disclosure fully complies with the requirements set forth in 37 C.F.R. § 1.821 to § 1.825. Pursuant to 37 C.F.R. §1.821 (g), the undersigned attorney hereby states that this submission does not contain new matter. Pursuant to 37 C.F.R. §1.821 (f), the undersigned attorney hereby states that the content of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R. §1.821 (c) and (e), respectively, are the same.

Claims 9 and 11-15 have been amended; claims 9 and 11-16 are pending in this application. No new matter is added.

It is submitted that the claims, herewith and as originally presented, are patentably distinct over the prior art cited by the Examiner, and that these claims were in full compliance with the requirements of 35 U.S.C. §112. The amendments of and additions to the claims, as presented herein, are not made for purposes of patentability within the meaning of 35 U.S.C. §§§§ 101, 102, 103 or 112. Rather, these amendments and additions are made simply for clarification and to round out the scope of protection to which Applicants are entitled. Support is found throughout the specification and from the pending claims.

Objections to the Specification and Claims

The specification was objected to as containing essential material improperly incorporated by reference. The material from Wasmann *et al.* (copy attached hereto) has been added to the specification by this amendment. Attached is a declaration according to MPEP 608.01(p) I.A.2, executed by the undersigned, stating that the amendatory material consists of the same material incorporated by reference in the application. No new matter is added by this amendment.

Claims 9, 12, 14 and 15 were objected to because of certain informalities. These claims have been amended to address and overcome each of these objections. Reconsideration and withdrawal of the claim objections is requested.

The Rejections Under 35 U.S.C. §112, 2nd Paragraph, Are Overcome

Claims 9 and 11-16 were rejected under 35 U.S.C. §112, second paragraph, as being indefinite. Claims 9 and 11-14 have been amended as suggested by the Examiner. The rejection of claim 15 (and dependent claim 16) is traversed. Page 8, lines 11-13 of the specification states

that, "[t]he nucleic acid segments or constructs can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of *A. tumefaciens*." The state of the art use of such Ti plasmids is specified in the paragraphs of the specification that follow, particularly the paragraph bridging pages 8 and 9. A "vector" is known to the skilled artisan as a "DNA molecule which serves as a recipient or carrier for foreign DNA. Vectors are usually plasmid or phase DNA molecules which carry an origin of DNA replication and genetic markers which allow them to be detected in host cells." (See E. Winnacker, 1987, From Genes to Clones, VCH Verlagsgesellschaft mbH, 198'7, Appendix C, page 540; copy attached.)

Claim 15 is distinct from claim 14 because the introduction of foreign nucleic acid molecules into a plant genome can be managed by various techniques. Plant cells can be transformed by protoplast transformation, microinjection, electroporation, or biolistics, without using a vector molecule (see application at page 9, paragraph beginning at line 10), or by using vectors like Ti vectors (see paragraph bridging pages 8 and 9 of the specification).

Applicants request that the various rejections under 35 U.S.C. §112, second paragraph, be reconsidered and withdrawn in light of these amendments and arguments.

The Rejections Under 35 U.S.C. §112, 1st Paragraph, Are Overcome

Claims 14-16 were rejected under 35 U.S.C. §112, first paragraph, as allegedly reciting new matter that is broader in scope than that recited in the specification. Applicants respectfully disagree and submit that Example 1 contains all the information that is needed by a skilled artisan in order to clone the sequence from the source plasmid described in the referenced literature (page 15, line 3), the essential elements of which have been added by this amendment. All procedures for the excision of the nucleic acid sequence encoding the transit peptide sequence from the small subunit of the pea Ribulosebisphosphate Carboxylase, including the restriction enzymes (*Hin*d 3 and *Sph* 1), and for the fusion of the nucleic acid sequence with the ASN-A-encoding nucleic acid molecule are sufficiently disclosed.

Furthermore, the amino acids are given in the primary literature as cited (Wasmann et al., 1986) and now recited in the specification as a result of this amendment. The method for sub-cloning the sequence from the pNi6/25 vector is exactly defined, as are the ultimate characteristics of the fragment. Any skilled molecular biologist would be able to follow these instructions, which are absolutely sufficient to clone the ASN-A gene sequence and transit peptide, and to further insert this sequence into the pDH51 vector (page 15, lines 6-9). Any other

transit peptide can be used in lieu of the pea sequence, as there are conservative elements, which may differ slightly in size but are common in function, and these elements are annotated in known nucleic acid/protein databases. The skilled plant molecular biologist would be able, without undue experimentation, to fuse a transit peptide from any source (numerous sequences were publicly available at the time of filing) with an ASN-A-encoding nucleic acid sequence, either by applying the respective restriction endonucloases or by re-synthesizing such a short nucleic acid molecule encoding the transit peptide *in vitro* (Climie, S. *et al.*, (1990), Chemical synthesis of the thymidylate synthase gene, Proc. Natl. Acad. Sci. USA, 87, 633-637; Italzura, *et al.*, (1984), Synthesis and use of synthetic oligonucleotides, Ann. Rev. Biochem., 53, 323-356).

Claims 9 and 11-16 were rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. The arguments refuting the written description rejection above apply to this rejection as well. The guidance present in the specification permits the cloning of further asparagine synthetase genes by the skilled artisan using standard plant molecular biology tools. If not available as a cloned nucleic sequence, such a gene can also easily be synthesized *in vitro* by applying standard means, as described in the preceding paragraph. For example, page 6, numbered points 1-3, briefly explains how the skilled artisan may proceed in order to identify further ASN-A genes from other organisms for making transgenic plants. The specification contains clear teachings on which classes of nucleic acid fragment to clone (tp and ASN) and methods for cloning them in order to obtain transgenic plants that exhibit the described features.

The Rejections Under 35 U.S.C. §103(a) Are Overcome

Claims 9, 11-13 and 16 were rejected under 35 U.S.C. §103(a), as allegedly being unpatentable over Coruzzi *et al.* (AG), in view of Dudits *et al.* (AH), Temple *et al.* (AP), and Della-Cioppa *et al.* Applicants urge that none of the cited references, either alone, or in any fair combination, serve to teach or suggest the presently claimed invention.

Applicants respectfully disagree with the argument bridging pages 14 and 15 of the Office Action that the originally filed claims don't limit the anti-sense constructs to chloroplastic GS. Amended claims 9 and 11 address this point in that the transfer and integration of an anti-sense chloroplastic glutamine synthetase sequence (claim 9), as well as its reduced level of expression, of the chloroplastic glutamine synthetase, specifically, (claim 11) are claimed. The amendment is supported by examples 4 and 5 of the specification, in which the inhibition of chloroplastic glutamine synthetase, specifically, is addressed in various plants. Moreover, there

is no Example or any detailed description in the specification regarding how to (successfully) reduce the expression level of GS_1.

Within the specification, it is disclosed that "[t]he methods can further comprise treating the plant with a glutamine synthetase inhibitor" (paragraph bridging pages 5 and 6). GS inhibitors, such as L-Phosphinothricin (glufosinate ammonium) are active in the photosynthetic tissues of the plant in order to inhibit the GS activity. As is shown, for example, in the attached graph (Figure 8.8 from Buchanan *et al.*, 2000, Biochemistry and Molecular Biology of Plants: 367), GS_2 is localized in the chloroplast with high specificity. Therefore, it makes sense to address a GS_2-specific reduction, as was done in Examples 4 and 5 of present specification. Figure 8.8 clearly points out that only the GS_2 is the relevant GS to be addressed by the present approach, as it is the only one showing significant activity in leaves.

The differences between GS_1 and GS_2 are also described on page 366 of Buchanan *et al.* (attached). Although this textbook is published later than the filing date of present application, it clearly demonstrates the differences accepted in the art between GS_1 and GS_2. Further, the same view was expressed by Li *et al.* with regard to maize GS_1 in 1993 (Plant Molecular Biology, 23, 401-407): "The transcript of the putative chloroplastic GS_2 gene was found to accumulate primarily in green tissues as expected, although some transcript was detected in roots" (at 402, bottom of column 2); and "... GS2 transcript accumulates preferentially in green tissues" (at 404, column 2). Li *et al.* also reported that "[t]he coding regions of the five new cytosolic-like GS cDNAs exhibit from 80 to 96% nucleotide sequence identity when compared with each other and 67 to 70% nucleotide sequence identity when compared with the homologous regions (excluding the putative transit peptide coding region) of the GS_2 cDNA" (at 402, column 1).

These significant differences in sequence identity clearly point out that a selective repression is possible through inhibition of the target RNA using the right anti-sense sequence.

The Office Action states in the last lines of page 15 that "Coruzzi clearly teaches the importance of targeting GS_2." However, the section of Coruzzi cited in the Office Action (page 29, lines 26-29) reads: "In embodiments, where suppression of most, if not all, GS isozymes is desired, it is preferred that the co-suppression construct encodes a complete or partial copy of chlororplastic GS mRNA (e.g., pea GS2 mRNA)."

Coruzzi refers to the suppression of more than one (if not all, in a non-selective manner) GS, which is, according to the disclosure, at least directed to cytosolic GS_1 and GS_3, as well as

chloroplastic GS_2. This again clearly demonstrates that Coruzzi is not aware of the relevance of a selective knockout of the chloroplastic GS_2 as described in the present application. In no case, including in section 6.2.2 of Coruzzi, is the combination of a reduction of GS_2 and the timely adjusted up-regulation of ASN reported. The only related information is on page 48, lines 24-28: "Typically, plants co-suppressed for GS2 grow more slowly than wild-type and developed intervenial chlorosis (see Figure 10) due either to the toxicity associated with ammonia accumulation during photorespiration, or glutamine deficiency." There is no teaching, suggestion or motivation toward the combination of suppression of chloroplastic GS_2 and an increased level of AS in a single transgenic plant. The combination of suppressed GS_2 with prokaryotic ASN-A, which uses ammonium rather than glutamine for the production of asparagine (Cedar and Schwartz (1969) J. Bioi. Chem. 244, 4112-4121) is also neither taught nor suggested. Contrary to this, Coruzzi reported on page 20, lines 22-28 the following:

"These plants having one altered enzyme also may be crossed with other altered plants engineered with alterations in the other nitrogen assimilation or utilization enzymes (e.g. cross a GS overexpressing plant to an AS overexpressing plant) to produce lines with even further enhanced physiological and/or agronomic properties compared to the parents."

Herein, Coruzzi *et al.* discuss simultaneous overexpression of GS and AS, but not about repression of GS and overexpression of AS.

Furthermore, Coruzzi *et al.* explain in more detail on page 21, lines 20-36:

"The present invention provides that engineering ectopic overexpression of one or more of those enzymes would produce plants with the desired physiological and agronomic properties. ... The engineering of enhanced expression of "root-specific" cytosolic GS (e.g., pea GS_1 is especially preferred." (Emphasis added. See also Table 2 on page 55 and Table 3 on pages 56/57 and related comments within the corresponding text.)

Coruzzi *et al.* actually teach away from the concept of the present invention. Consider the following statements: "these results show that the growth improvements are due to GS overexpression and not to the mere engineering of plants with the CaMV-35S GS constructs. For example, Z54-Al, which as been engineered with CaMV 35S-GS2 and was co-suppressed for GS expression, exhibited profoundly poor growth" (page 58, lines 4-12); and, "these results demonstrate that GS activity is a rate limiting step in plant growth as inhibition of his enzyme causes severe phenotypic effects on growth" (page 58, lines 9-12).

This is contrary to the present invention, where a reduction of GS activity is combined with the overexpression of a prokaryotic ASN in a transgenic plant, resulting in a significant increase in the observed tuber weight of the transgenic plants. Moreover, these transgenic plants show more vigorous growth and flower earlier than wild type plants.

According to Coruzzi's assumptions, the inventive concept of an GS reduction and AS overexpression, as claimed herein, should not work at all.

Concerning AS overexpression, Coruzzi *et al.* refer to the ectopic expression of AS in a transgenic plant (ectopic as explained/defined in the Coruzzi specification to mean "in all cell types", see page 62, lines 16-17). In addition, Coruzzi et al state that "the studies presented here examined whether the ectopic overexpression of AS in all cell types in a light-dependent fashion would increase asparagine production."

Contrary to this, the present specification clearly states and claims a prokaryotic AS (ASN) which either is targeted to the chloroplasts or is expressed directly in the chloroplasts. This clearly means that the overexpressed ASN is present only in chloroplasts, and thereby only in green tissue, not in all cell types, as disclosed and intended by Corazzi *et al*. Neither Coruzzi, Temple, Dudits, nor any combination of them teach or suggest that the combination of anti-sense GS_2 and overexpressed prokaryotic ASN would have a beneficial effect.

Coruzzi *et al.* further state: "It can therefore be anticipated that creating transgenic lines which express both GS and AS at high levels (by crossing AS and GS overexpressers) may have even more advantageous growth traits than either parent. In particular, the approaches disclosed here have the advantage that assimilation in transgenic lines will not be restricted to a few cell types, enabling available nitrogen in all plant cells to be utilized. The ectopic overexpression of both GS and AS in a single plant may have the advantage of avoiding glutamine accumulation..." (page 77, line 30 - page 78, line 8). Again, this is based on an increase in GS (which GS is not clear), rather than to a reduction in GS levels, either alone or in combination with overexpressed prokaryotic AS.

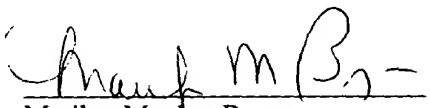
It is thus asserted that the claimed invention is not taught or suggested by the cited references, either individually or in any fair combination. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) are respectfully requested.

CONCLUSION

In view of the remarks and amendments herewith, the application is believed to be in condition for allowance. Favorable reconsideration of the application and prompt issuance of a Notice of Allowance are earnestly solicited. The undersigned looks forward to hearing favorably from the Examiner at an early date.

Respectfully submitted,

FROMMER LAWRENCE & HAUG LLP
Attorneys for Applicants

By: 

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification

On page 15, line 1:

The nucleotide sequence of the modified transit peptide from the small subunit of Ribulosebiphosphate Carboxylase from pea was isolated from the vector pNi6/25 (Wasmann, C.C. et al (1986) Mol. Gen. Genet. 205: 446-453) as a Hind3/Sph1 fragment. As described by Wasmann et al., the pNi6/25 vector was derived by cloning *Eco*RV-*Bam*HI fragments containing the modified transit peptide sequence into a vector fragment produced from ptac/TPNPTII by digestion with *Eco*RV and *Bam*HI. The ptac/TPNPTII vector was derived from pTPK1, which was constructed by ligating an *Eco*RI-*Bam*HI vector fragment from pKM109/15 with the *Hin*dIII-*Bam*HI fragment of pTP2 that contains the transit peptide coding sequence and the *Eco*RI-*Hin*dIII fragment of ptac12/Hind that carries the *tac* promoter. pKM109/15 contains the NPTII gene with an upstream *Bam*HI site. Plasmid pTP2 was derived from pTP1, which carries the *Eco*RI-*Sph*I fragment of pPSR6 (Cashmore, (1983) In: Genetic engineering of plants - An agricultural perspective; Ed. Kosuge et al. Plenum Publishing, NY, pp. 29-38) that codes for the promoter and transit peptide of the small subunit in pBR327 (Soberon et al. (1980), Gene 9:287-305). [This]The modified transit peptide (SEQ ID NO: 3) contains a duplication of 20 amino acids compared to the natural transit peptide (SEQ ID NO: 4). The 20 amino acid duplication results in increased transport into chloroplasts over that observed with the natural transit peptide (Wasmann et al.).

In the Claims

9. (Thrice amended) A process for the production of plants with improved growth characteristics, which comprises the following steps:

b) transferring and integrating[integration of] a nucleic acid[DNA sequence] encoding a polypeptide coding region comprising[for] a prokaryotic asparagine synthetase coding region[in the plant genome ; wherein said DNA sequence is] linked to a [regulatory]chloroplast leader sequence for [the expression of said DNA sequence and] import of the asparagine synthetase into [the] chloroplasts or plastids of a plant cell, [and] wherein said nucleic acid is operatively linked to a regulatory sequence for expression in said plant cell [exhibits the biochemical activity of the imported asparagine synthetase in its chloroplasts or plastids];

b) transferring and integrating[integration of] a nucleic acid for expression of an antisense chloroplastic glutamine synthetase RNA[gene] or [a] portion thereof comprising transferring and integrating[into the plant genome which encodes and expresses] an anti-sense chloroplastic glutamine synthetase nucleic acid operatively[RNA of said gene wherein the DNA sequence is] linked to a regulatory sequence for expression of said anti-sense RNA or portion thereof in said cell to make a transformed cell;[the transcription of said DNA sequence] and

c) regenerating[regeneration of] intact and fertile plants from the transformed cells.

11. (Thrice amended) A plant cell obtainable by the[a] method [as claimed in]of claim 9, comprising:

a) a nucleic acid encoding a polypeptide coding region comprising a prokaryotic asparagine synthetase coding region linked to a chloroplast leader sequence for import of the asparagine synthetase into chloroplasts or plastids of a plant cell, wherein said nucleic acid is operatively linked to a regulatory sequence for expression in said plant cell; and

b) a second nucleic acid for expression of an anti-sense RNA to an endogenous chloroplastic glutamine synthetase gene or portion thereof comprising a nucleic acid comprising an endogenous chloroplastic glutamine sythetase or portion thereof in an anti-sense orientation operatively linked to a regulatory sequence,

said second nucleic acid providing reduced levels of endogenous chloroplastic glutamine synthetase activity upon expression of said anti-sense RNA in said cell [wherein a prokaryotic ammonium specific asparagine synthetase exhibits the biochemical activity of the imported asparagine synthetase in its chloroplasts or plastids and which contains a gene construct which provides a reduced level of expression of endogenous chloroplastic glutamine synthetase activity].

12. (Amended) A plant, seed[s,] or propagule [or propagation material] containing a cell[s] according to claim 11.

13. (Twice amended) A gene construct comprising a nucleic acid[gene] encoding a polypeptide coding region comprising a prokaryotic ammonium specific asparagine synthetase coding region [operatively] linked to a chloroplastic leader sequence for [regulatory sequence for the expression of said gene and] import of the asparagine synthetase into the chloroplasts or plastids of a plant cell, and which construct is operatively linked to a regulatory sequence for expression in said plant cell, and wherein said plant cell exhibits the biochemical activity of the imported asparagine synthetase in its chloroplasts or plastids.

14. (Twice amended) A gene construct according to claim 13, wherein the prokaryotic asparagine synthetase polypeptide coding region is linked[gene is an E. coli asparagine synthetase gene with a chloroplasic leader peptide] at its N-terminus to[and which leader peptide is] a modified transit peptide coding region from[form] the small subunit of the Ribulosebisphosphate carboxylase from pea comprising[containing] a duplication of 20 amino acids from said[compared to the natural] transit peptide coding region.

15. (Thrice amended) A vector comprising[containing a] the gene construct according to claim 13[14 which gene construct comprises a sequence which encodes a chloroplastic leader peptide at its N-terminus and which leader peptide is a modified transit peptideform the small subunit of the Ribulosebisphosphate carboxylase from pea containing a duplication of 20 amino acids compared to the natural transit peptide].

16. (Amended) A plant cell transformed with the gene construct according to claim 13 or 14, or with the vector according to claim 15.

#16
attach

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and are engaged in splicing of heterogenous nuclear RNA to mRNA.

Somatic cells All cells of a higher organism which are not reproductive cells (eggs or spermatozoa).

Splicing Process of conversion of primary RNA transcripts to mature and translatable mRNA. Since many genes are split, *i.e.* since they contain introns and exons, primary transcripts also contain sequence regions which do not code for proteins. Such sequences are removed from the primary transcript while the ends of the coding regions are correctly fused (spliced).

Superhelix Tertiary structure of double-stranded circular DNA molecules which is characterised by supertwists introduced into these molecules.

Suppressor tRNA molecule which directs the incorporation of an amino acid at the position of a stop codon. Suppressors have been found in *E. coli* and *Saccharomyces cerevisiae*.

Temperate phage Class of bacterial viruses which do not only undergo a lytic growth cycle but are also capable of integrating a copy of their genome into the host cell. Host cells which contain an integrated bacteriophage are called lysogens.

Terminator Sequence region on a DNA molecule which signals the termination of transcription.

Transcript RNA copy of a DNA region.

Transcription Process of RNA synthesis from a double-stranded DNA as template.

Transcription unit DNA region which comprises exons, introns and all other control elements required for gene expression.

Transfection (*cf.* also transformation) Both terms describe the transfer of pure DNA into intact viable cells. Transfection is usually used for manipulations involving higher cells. Transformation in higher cells usually denotes the transition from a normal to a tumour phenotype.

Transformation Transfer of purified DNA, recombinant DNA, for example, to bacteria or higher cells.

Transient expression Short-lived expression of a gene from vectors which are introduced into a non-permissive cell. Since such vectors cannot replicate, the copy numbers gradually decrease upon cell division with a concomitant reduction in gene expression.

Translation Formation of protein molecules with mRNA as template.

Transposon Movable genetic element which is capable of jumping from one chromosome to another chromosome. Structurally transposons are genes which are flanked by identical nucleotide sequences in opposite orientation.

Tumour antigen Protein which is specifically found in tumour cells or transformed cells and which can be recognised by immune reactions with sera from tumour-bearing animals.

Vector DNA molecule which serves as a recipient or carrier for foreign DNA. Vectors are usually plasmid or phage DNA molecules which carry an origin of DNA replication and genetic markers which allow them to be detected in host cells.

Z-DNA Alternative left-handed form of the DNA double helix which retains Watson-Crick-type base pairing.

Zygote Single cell obtained by fusion of an egg cell with a spermatozoon. The genes of a zygote represent a random assortment of genes from both parent cells.

#16 attach

Cover: Portion of the ribbon structure of dinitrogenase (MoFe-protein); see Figure 16.7, adapted with permission from Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B., Rees, D. C. 1997. Structure of ADP•AlF_4–stabilized nitrogenase complex and its implications for signal transduction. *Nature* 387: 370–376. 1997 Macmillan Magazines Ltd.

Part-title pages
Part 1: Iceplant (*Carpobrotus chilensis*) at sunset, coastal bluffs of Marin Headlands, California, 1991; Part 2: Spring wildflowers on Vollmer Peak, Tilden Regional Park, Berkeley, California; Part 3: Sunbeams in morning fog, Berkeley, California, 1996; Part 4: Coast redwood, Big Basin Redwoods State Park, California, 1995; Part 5: The High Sierra as seen from the Central Valley, near Madera, California, on a clear winter day. Used with permission from Galen Rowell/Mountain Light Photography, Emeryville, California.

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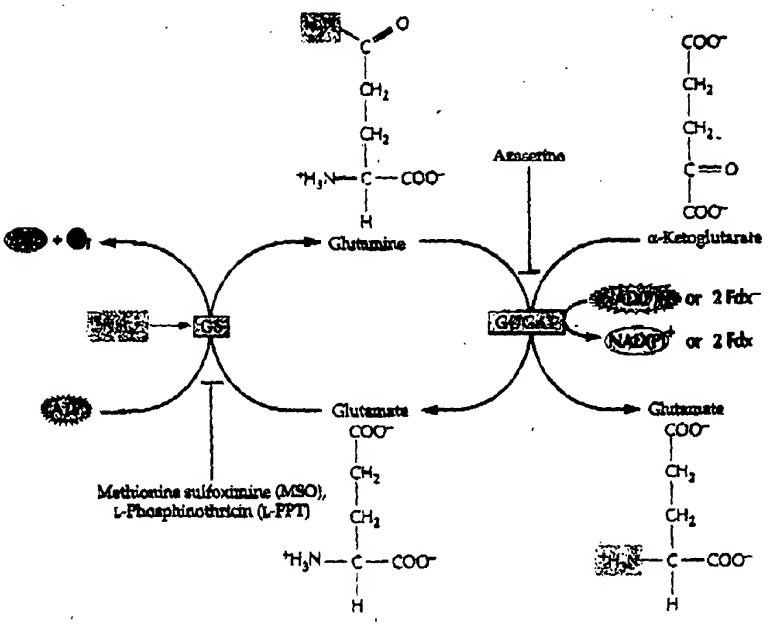

Figure 8.5
The glutamine synthetase–glutamate synthase (GS/GOGAT) pathway is thought to be the principal mechanism of primary and secondary ammonium assimilation. Sites of action are shown for several enzyme inhibitors. Fdx, ferredoxin.

8.2.2 Molecular and genetic studies demonstrate that cytosolic and chloroplast GS isoenzymes perform nonoverlapping roles in vivo.

GS isoenzymes can be separated into two classes by ion-exchange chromatography—one localized in the cytosol (GS1), the other in the chloroplast (GS2). Genes encoding each GS isoenzyme have been cloned by several different methods, including cross-hybridization to animal *GS* cDNAs and complementation of microbial *GS* mutants. Plant *GS* cDNAs are able to complement bacterial *GS* mutants, despite the fact that their respective holoenzymes are distinct in subunit structure and sequence. Plants examined thus far appear to possess a single nuclear gene encoding GS2 and multiple (two to four) nuclear genes encoding GS1 subunits. The GS holoenzymes in plants function as octamers, and GS1 polypeptides can assemble into homo- or heterooctamers. Although the chloroplast and cytosolic GS holoenzymes do not appear to differ significantly in their biochemical properties when assayed

Box 8.2

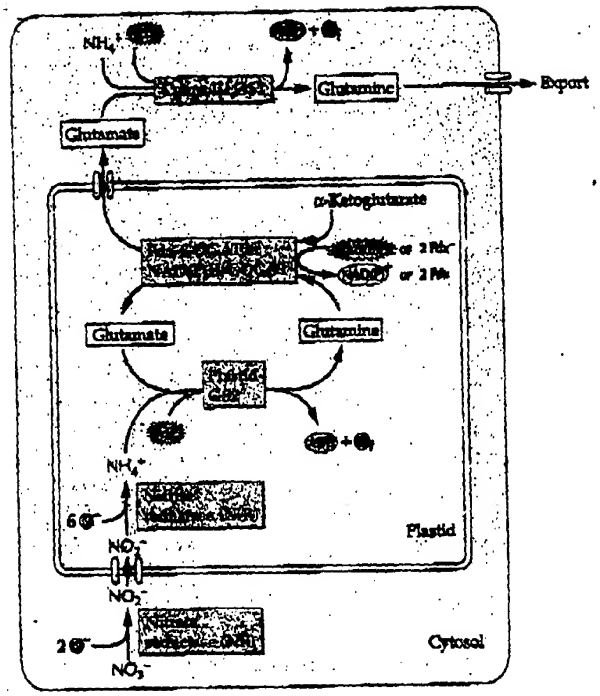

Figure 8.6
Isoenzymes of glutamine synthetase (GS) are present in both the plastids (GS2) and the cytoplasm (GS1). Fdx, ferredoxin.

in vitro, they have distinct in vivo functions. The chloroplast GS2 holoenzyme is the predominant GS isoenzyme in leaves (Fig. 8.8), where it is thought to function both in primary ammonia assimilation and in the reassimilation of photorespiratory ammonia. Cytosolic GS1 isoenzymes are present at low concentrations in leaves and at higher concentrations in roots, suggesting that this isoenzyme has a role in primary assimilation in roots. In some nitrogen-fixing legumes, nodule-specific cytosolic GS isoenzymes (termed GSn) assimilate nitrogen fixed by rhizobia (see Chapter 16).

The proposed roles of GS isoenzymes inferred from their organ-specific distribution have been refined by more recent molecular and genetic analysis. Genes encoding chloroplast and cytosolic GS isoenzymes are expressed in distinct cell types. The gene for chloroplast GS2 is expressed in mesophyll cells, whereas the genes for cytosolic GS1 isoenzymes appear to be expressed specifically in phloem (Fig. 8.9). These distinct cell-specific patterns of gene expression suggest that the chloroplast and cytosolic GS isoenzymes perform nonoverlapping functions in vivo. The phloem-specific GS1 probably synthesizes glutamine for long-distance nitrogen transport. In contrast, the specific expression of chloroplast GS2 in mesophyll cells indicates a role for this isoenzyme in primary nitrogen assimilation, or in the reassimilation of photorespiratory ammonium. Genetic evidence supports the latter conclusion. First, mutants in chloroplast GS2 display a conditional lethality: They die in air but grow in a 1% CO_2 atmosphere that suppresses photorespiration. Thus, the chloroplast GS2 enzyme is responsible for reassimilating photorespiratory ammonium released in the mitochondria. This example demonstrates how a mutant can be used to define the flux through a pathway in vivo when the parameters regulating intra- and intercellular transport are unknown. Second, the GS2 mutants have normal amounts of cytosolic GS1 (see Fig. 8.8), confirming that the GS isoenzymes play nonoverlapping roles. Moreover, the phloem-specific expression of

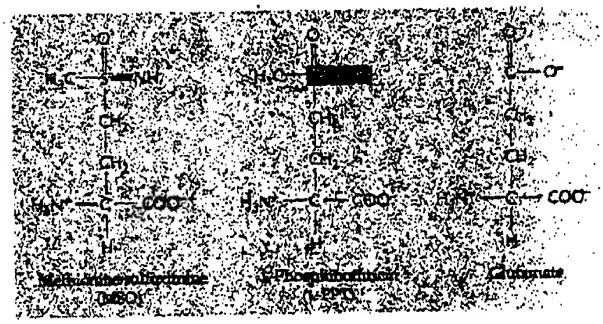

Figure 8.7
Methionine sulfoximine and phosphinothricin, competitive inhibitors of glutamine synthetase.

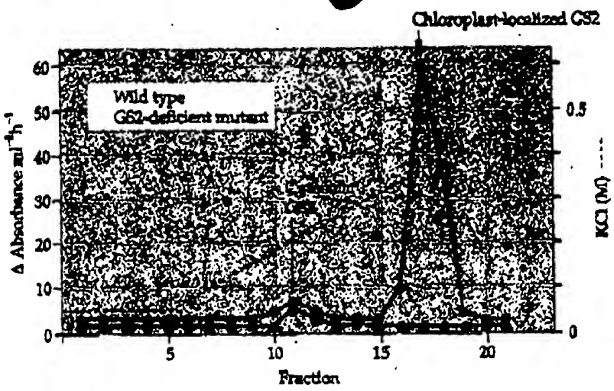

Figure 8.8
The plastid-localized isoenzyme GS2 is the principal source of GS activity in leaves. Shown are the results of separating GS2 and cytosolic GS1 by ion-exchange chromatography. Desalted extracts from young, expanding leaves were applied to an ion-exchange column and eluted with a linear KCl gradient (dotted line).

cytosolic GS1 may explain why this isoenzyme is unable to compensate for the loss of the chloroplast GS2 isoenzyme in leaf mesophyll cells of the barley GS2 photorespiratory mutants (Box 8.2).

8.2.3 Mutants indicate a major role for Fdx-GOGAT in photorespiration.

Despite extensive biochemical characterizations of Fdx-GOGAT and NADH-GOGAT in plants, it is not currently resolved whether these isoenzymes perform overlapping or distinct functions in vivo. Quantitative analyses of each isoenzyme in various tissues have been used as circumstantial evidence to propose an in vivo role. For example, Fdx-GOGAT is the predominant GOGAT isoenzyme in leaves and can account for as much as 95% to 97% of total leaf GOGAT activity, as determined in *Arabidopsis* and barley (Table 8.1). In contrast, the NADH-GOGAT isoenzyme is present in low amounts in leaves but constitutes the predominant isoenzyme in nonphotosynthetic tissues such as

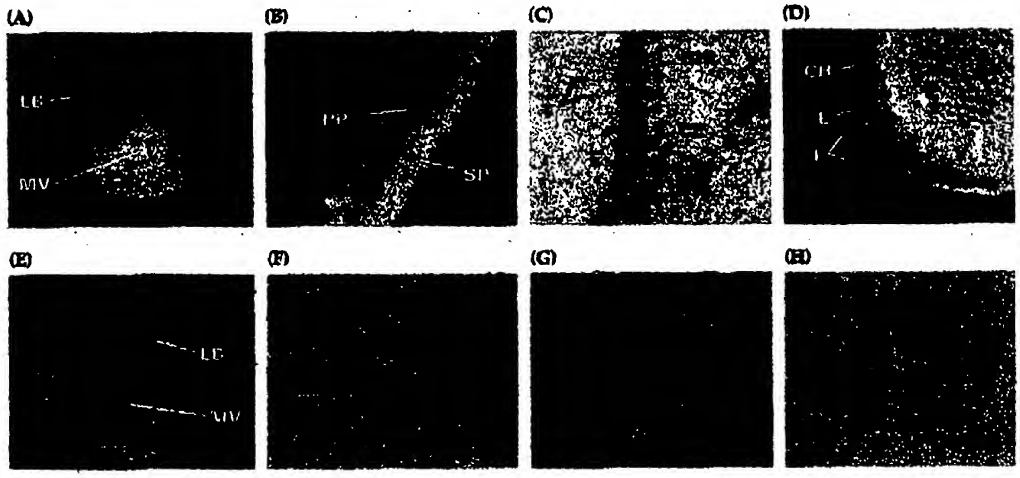

Figure 8.9
Photographs and light micrographs of mature transgenic tobacco plants show that GS1 and GS2 promoters are expressed in different tissue types. Use of β-glucuronidase staining to analyze promoter-specific expression patterns is described in Chapter 7, Box 7.5. Here, the promoter for chloroplast-localized GS2 is expressed in photosynthetic cells (A–D), whereas the promoter for cytosolic GS1 is expressed in phloem. CH, chlorenchyma; CL, collenchyma; E, epidermis; LB, leaf blade; MV, midvein; PH, phloem; PP, palisade parenchyma; PT, pith parenchyma; R, root; SP, spongy parenchyma; T, trichome; V, vasculature; X, xylem.

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Update section

Short communication

Differential expression of six glutamine synth

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Key words: gene-specific probes, glutamine synthetase, transcript accumulation, *Zea mays*

Abstract

The maize genome has been shown to contain six glutamine synthetase (GS) genes with at least four different expression patterns. Noncoding 3′ gene-specific probes were constructed from all six GS cDNA clones and used to examine transcript levels in selected organs by RNA gel blot hybridization experiments. The transcript of the single putative chloroplastic GS_2 gene was found to accumulate primarily in green tissues, whereas the transcripts of the five putative GS_1 genes were shown to accumulate preferentially in roots. The specific patterns of transcript accumulation were quite distinct for the five GS_1 genes, with the exception of two closely related genes.

Glutamine synthetase (GS; EC 6.3.1.2) plays a central role in the flow of nitrogen into nitrogenous organic compounds of plant cells by catalyzing the assimilation of ammonia into glutamine, which is then converted to glutamate via the action of glutamate synthase [13]. In higher plants, GS is known to occur as multiple isoenzymes [6] with one isoenzyme (designated GS_2) localized in chloroplasts and one or more isoenzymes (designated GS_1) present in the cytosol [12]. The GS isoenzymes of plants are encoded by small gene families [2, 3], with the largest GS gene family reported prior to this study being the five GS genes in *Phaseolus vulgaris* [6]. In this paper, we present results showing that the maize genome contains six different GS genes that exhibit five distinct patterns of transcript accumulation.

A maize inbred A188 seedling cDNA library was prepared in λgt10 and screened for GS-coding sequences by *in situ* plaque hybridization with the putative chloroplastic GS cDNA [21] as probe. A single positive putative GS_1 cDNA clone was identified and used as a probe to rescreen the library. Characterization of the restriction pat-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X65926 (GS_{1-1}), X65927 (GS_{1-2}), X65928 (GS_{1-3}), X65929 (GS_{1-4}), X65930 (GS_{1-5}) and X65931 (GS_2).

termus of the positiv clones isolated in the rescreen demonstrated the presence of four new GS sequences. One cDNA clone of each type was subcloned in pUC118 and pUC119 [25], and nested sets of deletion subclones were constructed and sequenced by the dideoxynucleotide chain termination procedure [19]. Based on sequence similarities with GS-coding sequences of other species, the five new cDNAs appear to encode GS polypeptides of the cytosolic type. Thus, they have been designated $GS_{1\text{-}1}$, $GS_{1\text{-}2}$, $GS_{1\text{-}3}$, $GS_{1\text{-}4}$, and $GS_{1\text{-}5}$ and the previously characterized maize GS cDNA [21], putatively encoding chloroplastic GS, has been designated GS_2. All of the cDNAs contain complete coding sequences with the exception of $GS_{1\text{-}5}$, which is lacking part of the 5'-coding sequence. The 5' terminus of GS_2 was resequenced during the present investigation, and one error in the GS_2 sequence published by Snustad *et al.* [21] was detected. The corrected sequence predicts a GS polypeptide with a putative chloroplast transit peptide 41 amino acids in length with striking similarity to the predicted transit peptide of the GS_2 polypeptide of rice [18].

The coding regions of the five new cytosolic-like GS cDNAs exhibit from 80 to 96% nucleotide sequence identity when compared with each other and 67 to 70% nucleotide sequence identity when compared with the homologous regions (excluding the putative transit peptide coding region) of the GS_2 cDNA. There are two pairs of closely related cDNAs. The coding sequences of $GS_{1\text{-}1}$ and $GS_{1\text{-}5}$ (partial) share 96% nucleotide sequence identity, and those of $GS_{1\text{-}3}$ and $GS_{1\text{-}4}$ exhibit 94% nucleotide sequence identity. Five of the A188 GS cDNAs appear to be very similar, but not identical, to the GS cDNAs from maize cv. Golden Cross Bantam T51 characterized by Sakakibara *et al.* [17], with from 3 to 11 nucleotide differences in the coding regions, respectively. Clones pGS122, pGS112, pGS107, and pGS117 of Sakakibara *et al.* correspond to cDNAs $GS_{1\text{-}1}$, $GS_{1\text{-}3}$, $GS_{1\text{-}4}$, and $GS_{1\text{-}5}$ respectively, described here. The sixth gene, $GS_{1\text{-}2}$, is not represented in the cDNA clones studied by Sakakibara *et al.*.

To relate our cDNA clones to specific genomic restriction fragments, we have constructed 3'-noncoding gene-specific hybridization probes for each of the GS_1 cDNAs and used these probes in a Southern blot analysis of the maize genome. In each case, the gene-specific probe hybridized to a single genomic restriction fragment, one of the set of restriction fragments detected with the GS_1-coding sequence probe (Fig. 1). Collectively, the GS_1 gene-specific probes detected all of the genomic fragments identified with the GS_1-coding sequence probe except for two (*Eco* RI-digested DNA) or three (*Hind* III-digested DNA) small fragments. Since these small fragments probably resulted from restriction sites within introns of the respective genes, the Southern blot results are consistent with a maize six-member GS gene family with each of our cloned cDNAs representing one member of the gene family. However, we cannot rule out additional GS genes on comigrating fragments or with divergent coding sequences.

To examine the sizes of individual GS gene transcripts and to obtain some information about the patterns of expression of the six identified GS genes, poly(A)$^+$ RNAs were isolated from a variety of organs/tissues and analyzed by northern blot hybridizations performed using both coding sequence and gene-specific probes. Figure 2 shows representative northern blot results demonstrating the distinct sizes of the GS_1 and GS_2 transcripts and showing the two very different patterns of transcript accumulation observed. The northern blot shown in Fig. 2 was stripped of radioactive probe and hybridized sequentially to each of the GS_1 3'-noncoding gene-specific probes. The results (not shown) demonstrated that all five GS_1 transcripts are ca. 1.4 kb long, whereas the size of GS_2 transcript is about 1.5 kb. The transcripts of two genes, $GS_{1\text{-}3}$ (Fig. 2) and $GS_{1\text{-}4}$ (data not shown), were observed to accumulate to detectable levels in most of the organs/tissues examined, whereas the transcripts of the other three GS_1 genes were observed to accumulate primarily in roots (Fig. 2, data shown only for $GS_{1\text{-}1}$). The transcript of the putative chloroplastic GS_2 gene was found to accumulate primarily

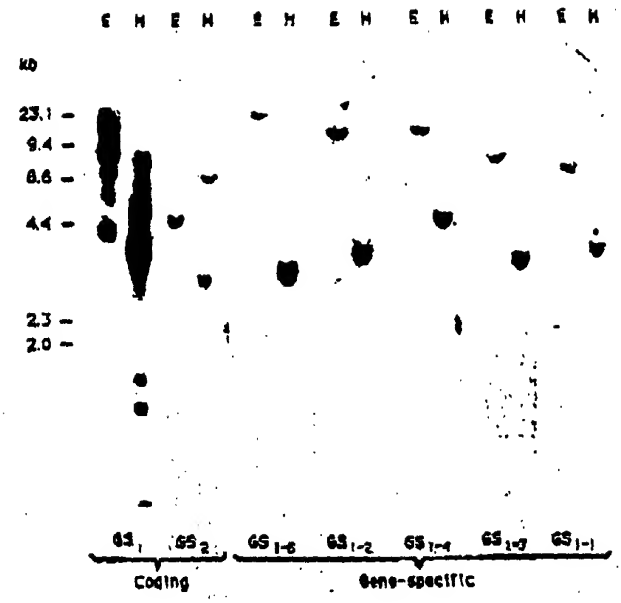

Fig. 1. Genomic Southern blot hybridization analysis of the GS genes of maize. Total DNA (15 μg samples) from inbred line A188 was digested with *Eco* RI (E) and *Hind* III (H), respectively. Hybridization and wash conditions were as described by Oppenheimer *et al.* [16]. The DNA fragments on a single blot were hybridized sequentially with the following radioactive probes: the GS_{1-1} cDNA minus the poly(A) tail, the GS_2 cDNA minus the poly(A) tail, and each of the five GS_1 3′-noncoding gene-specific probes. All probes were either single-stranded template DNAs with the desired sequences present in pUC118 or pUC119 or agarose gel-purified double-stranded insert DNAs excised from either pUC118 or pUC119. Single-stranded probes were ^{32}P-labeled by the primer-extension protocol of Hu and Messing [9]; double-stranded probes were ^{32}P-labeled by the random-primer protocol of Feinberg and Vogelstein [5]. The gene-specific probes were subclones containing almost exclusively 3′-noncoding sequences extending from restriction sites or deletion subclone termini near the translation-termination triplet to the 3′ termini of the cDNA clones. The six gene-specific probes contained the following sequences: GS_{1-1}, a 256 bp fragment with the 5′ terminus at a *Xho* I site 21 bp 3′ of the TGA; GS_{1-2}, a 170 bp fragment with the 5′ end at a *Sac* II site 7 bp 3′ of the TGA; GS_{1-4}, a 177 bp fragment with the 5′ end at a *Sac* II site 8 bp 3′ of the TGA; GS_{1-6}, a 272 bp fragment with the 5′ terminus at an *Xba* I site 67 bp 3′ of the TGA; GS_{1-7}, a 127 bp fragment with the 5′ end at a *Sph* I site 31 bp 3′ of the TGA; and GS_2, a 217 bp fragment with 5′ end at a deletion subclone terminus 49 bp 5′ of the TGA. The positions of marker DNA fragments of the indicated sizes are shown on the left.

in green tissues as expected, although some transcript was detected in roots (Fig. 2).

To obtain a more accurate picture of the differential accumulation of individual GS gene transcripts in organs of developing plants, we performed dot blot hybridizations using poly(A)$^+$ RNAs isolated from selected organs/tissues and quantitated the hybridized probes with a radioanalytic imaging system. Quantities of hybridized probe were normalized for poly(A)$^+$ RNA levels, estimated using labeled poly(U) as probe, in the RNA samples analyzed. This procedure facilitates comparisons of the levels of individual transcripts in different organs/tissues, but not quantitative comparisons of different transcript levels within organs/tissues. However, since the gene-specific probes were similar in size and were labeled using the same protocol, the results probably reflect, at a gross level, the levels of the different GS transcripts in the organs/tissues examined.

Since the results of the northern blot experiments indicated that all of the GS_1 genes were expressed in roots, we attempted to further local-

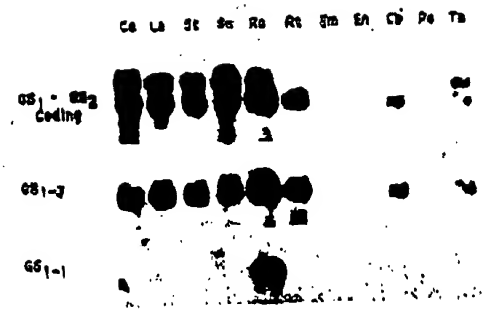

Fig. 3. Northern blot hybridization analysis of GS gene transcript accumulation in selected organs/tissues of maize. The poly(A)$^+$ RNAs (3.5 μg samples) on a single blot were hybridized sequentially with (top) a mixture of the GS_{1-1} and GS_2 cDNAs minus their poly(A) tails, (middle) a GS_{1-3} 3′-noncoding gene-specific probe, and (bottom) a GS_{1-1} 3′-noncoding gene-specific probe (see Fig. 1 legend for details on probes). When the RNA samples on this blot were hybridized with the GS_2 cDNA probe alone, only hybridizing bands corresponding in position to the upper bands present in the top photograph were observed (data not shown), indicating that the larger GS RNA species (about 1.4 kb in size) is the GS_2 transcript. The RNA samples were fractionated on formaldehyde-agarose gels [23] and transferred to nylon membranes (Nytran, Schleicher and Schuell) using 10 × SSC as the transfer solution. Hybridization and wash conditions were as described by Hussey *et al.* [10] except that 20 μg/ml poly(A) was added to the prehybridization and hybridization solutions. The RNAs are from Black Mexican Sweet culture cells (Cc), leaf blade (Lb), stem (St), seedling shoot (Ss), whole root (Ro), root tip (Rt), embryo (Em), developing endosperm (En), young cob (Cb), pollen (Po), and young tassel (Ts). The Black Mexican Sweet culture line was grown in Murashige and Skoog medium [15] supplemented with 2% (w/v) sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid, and 200 mg/l asparagine. All plant tissues were from inbred line A188. Seedling tissues were from six-day-old plants grown in the dark on wet filter paper at 25 °C. All other tissue/organ samples were from field-grown plants. Leaf blade and stem samples were from 30-day-old plants with the stem including one node and the adjacent internode. The seedling shoot included the first internode plus the coleoptile. Root tissue samples were from six-day-old seedlings. The root tip was defined as the 2 mm distal segment. Embryos and endosperms were dissected from ears 15 days after pollination; cobs were immature (1–2 cm in length) and unfertilized. Tassel primordia (0.5–1 cm in length) were dissected from four-week-old plants.

lize the individual GS transcripts within roots by dissecting roots of six-day-old seedlings into root tip, root cortex, and root vascular cylinder. The other organs/tissues investigated were stem, seedling shoot, leaf blade, immature cob, embryo, and pollen. The results of these dot blot experiments verified the results of the northern blot hybridizations and revealed some striking patterns of differential transcript accumulation within seedling roots. Figure 3 shows the results of dot blot hybridizations using probes specific for each of the six cloned GS cDNAs and poly(A)$^+$ RNAs isolated from the nine organs/tissues described above. In agreement with the northern blot data, the results of the dot blot hybridizations demonstrated that (1) the GS_{1-1}, GS_{1-2}, and GS_{1-5} transcripts accumulate preferentially in roots (Fig. 3A, B, and E), (2) the GS_{1-3} and GS_{1-4} transcripts accumulate to measurable levels in all nine organs/tissues examined and exhibit very similar patterns of transcript accumulation (Fig. 3C and D), and (3) the GS_2 transcript accumulates preferentially in green tissues (Fig. 3F).

The most striking result of the dot blot hybridization experiments was the differential accumulation of the root-preferential GS_{1-1}, GS_{1-2}, and GS_{1-5} transcripts within the cortical and vascular regions of seedling roots (Fig. 3A, B, and E). Whereas the transcript of the GS_{1-2} gene was found to accumulate primarily in the vascular tissues of roots, with a very small amount in the root cortex (Fig. 3B), the transcripts of genes GS_{1-1} and GS_{1-5} were shown to accumulate in the cortical tissues of seedling roots, with transcripts below the detectable level in the vasculature of roots (Fig. 3A and E). In addition to its high level of expression in root cortex, the GS_{1-5} transcript was also present in seedling shoot and in stem, two tissues where the GS_{1-1} transcript was barely detectable. Thus, with the exception of the closely related GS_{1-3} and GS_{1-4} genes, the six GS genes studied exhibited very different patterns of transcript accumulation in the organs/tissues investigated.

Sakakibara *et al.* [17] studied the effect of light and greening of tissues on five GS transcript levels in maize. Their results are consistent with those reported here, with only GS_2 transcript levels increasing during greening of tissues after transfer from dark to light. They did not analyze

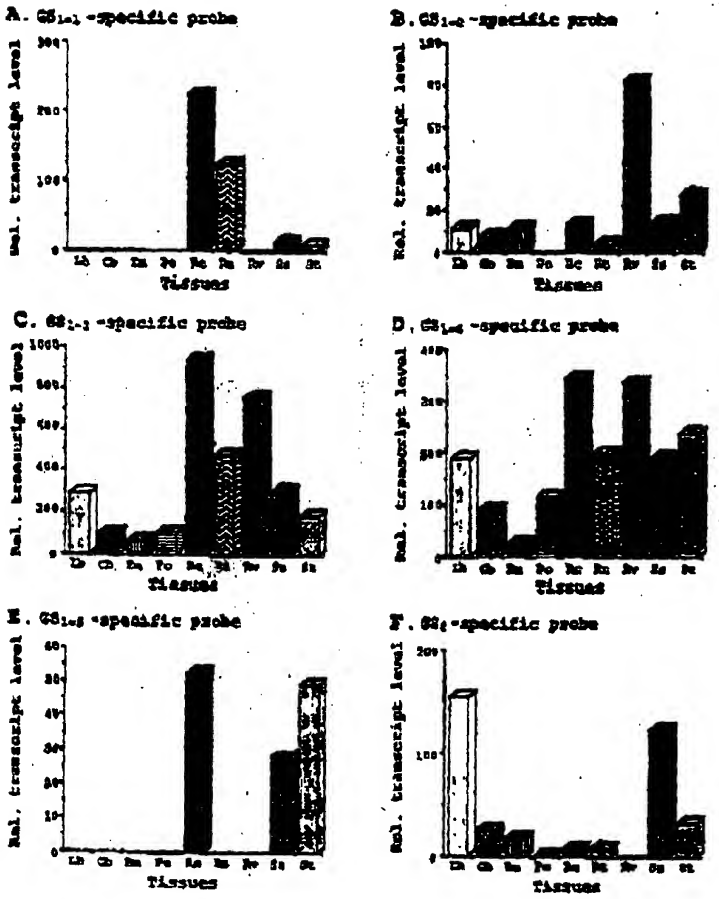

Fig. 3. RNA dot blot hybridization analysis of the accumulation of individual GS gene transcripts in selected tissues/organs of inbred line B73. The dot blots were prepared using poly(A)$^+$ RNAs as described by Joyce *et al.* [11]; for one tissue, root vascular cylinder, total RNA was used. RNA samples from two separate extractions were analyzed except for $GS_{1\text{-}4}$ and GS_2, and the results of one experiment are shown. The RNAs on the blots were hybridized with 3'-noncoding probes (see Fig. 1 legend) specific for each of the cloned GS cDNAs (A–F) using the conditions described by Oppenheimer *et al.* [16]. Hybridized probe was quantitated by radioanalyte imaging (Ambis, San Diego, CA), and the data were normalized by using the results of hybridization of the blots to labeled poly(U) probe as described by Joyce *et al.* [11]. This facilitates comparisons of the levels of a particular transcript in different organs/tissues, but does not permit valid comparisons of the levels of different transcripts, even within the same tissue. Relative transcript level is indicated on the ordinate of each graph. The organs/tissues analyzed were leaf blade (Lb), young cob (Cb), embryo (Em), pollen (Po), root cortex (Rc), root tip (Rt), root vascular cylinder (Rv), seedling shoot (Ss), and stem (St). The root cortex and root vascular cylinder samples were dissected from the length of six-day-old seedling roots excluding the 1-cm distal tips. All other organ and tissue samples were as described in the Fig. 2 legend.

the accumulation of the individual GS transcripts in different tissues and organs during plant development.

The differential expression of redundant GS genes during development has been documented for both *P. vulgaris* [1, 7, 8] and *P. sativum* [4, 24,

26]. In the present study, we have demonstrated that the six GS genes of maize exhibit five different patterns of expression. These results support the idea that the redundant GS genes of maize provide a mechanism for the differential regulation of GS synthesis during plant growth and development.

Yamaya and Oaks [27], Muhitch [14], and Ta [22] have proposed that the multiple GS isoenzymes may be involved in different aspects of nitrogen metabolism because the amide nitrogen of glutamine is used in a large number of metabolic pathways [20]. For example, glutamine is the major form of transported nitrogen in maize, and thus GS isoenzymes play a key role in the mobilization of nitrogenous compounds throughout the plant. In young seedlings, amino acids and small peptides obtained from the hydrolysis of endosperm proteins provide most of the nitrogen for growth. Seed formation also involves the transport of stored nitrogen from leaves and other parts of the plant to the developing kernels. During senescence, proteins in older leaves are hydrolyzed to provide nitrogen to younger leaves. Thus, it will be interesting to determine the expression patterns of the six GS genes of maize during these key developmental processes.

Given the redundancy of GS genes in maize, with six genes exhibiting five distinct patterns of transcript accumulation, a complete understanding of nitrogen assimilation in this important agronomic species will become available only when the specific functions of all of the GS gene products are known. The results reported here provide a good start toward achieving that objective.

Acknowledgements

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The importance of the transit peptide and the transported protein for protein import into chloroplasts

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Summary. We compared the transport in vitro of fusion proteins of neomycin phosphotransferase II (NPTII) with either the transit peptide of the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase or the transit peptide and the 23 aminoterminal amino acids of the mature small subunit. The results showed that the transit peptide is sufficient for import of NPTII. However, transport of the fusion protein consisting of the transit peptide linked directly to NPTII was very inefficient. In contrast, the fusion protein containing a part of the mature SSU was imported with an efficiency comparable to that of the authentic SSU precursor. We conclude from these results that other features of the precursor protein in addition to the transit peptide are important for transport into chloroplasts. In order to identify functional regions in the transit peptide, we analyzed the transport of mutant fusion proteins. We found that the transport of fusion proteins with large deletions in the aminoterminal, or central part was drastically reduced. In contrast, duplication of a part of the transit peptide led to a marked increase in transport.

Key words: Chloroplast protein transport – Mutants – NPTII fusions – Small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase – transit peptide

Introduction

The plant cell contains several membrane-bound organelles, the most conspicuous of which is the chloroplast. Most of the proteins of the chloroplast are encoded in nuclear DNA and synthesized in the cytoplasm. One example of a nuclear-encoded chloroplast protein is the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase. This chloroplast protein is synthesized in the cytoplasm as a higher molecular weight precursor possessing an amino-terminal extension, termed the transit peptide (Dobberstein et al. 1977). Transport of the SSU precursor (pSSU) is a post-translational (Chua and Schmidt 1978) and an energy-dependent process (Grossman et al. 1980). During or after transport of pSSU into the chloroplast, the transit peptide is removed enzymatically to yield the mature form of SSU (Ellis 1981; Schmidt and Mishkind 1986).

A fusion protein consisting of the transit peptide and the 23 aminoterminal amino acids of the mature SSU fused to neomycin phosphotransferase II (NPTII) is imported by chloroplasts in vivo (Schreier et al. 1985). Van den Broeck et al. (1985) have shown that a fusion of the transit peptide and NPTII is imported by chloroplasts both in vivo and in vitro. From their results, Van den Broeck et al. (1985) suggested that the transit peptide is sufficient for transport of a foreign protein into the chloroplast. However, because the transport efficiency of this fusion protein was not compared to that of either the authentic pSSU or the fusion protein of Schreier et al. (1985) it is still possible that other parts of the precursor play a role in transport.

In order to determine whether parts of mature SSU are important for chloroplast import, we compared the transport of two different NPTII fusion proteins and authentic pSSU into isolated intact chloroplasts. As was done previously by Van den Broeck et al. (1985), we assayed NPTII activity to detect the NPTII fusion proteins. A more quantitative and reliable comparison of transport efficiencies was obtained by using radioactively labeled precursor protein synthesized in vitro. We show that, although the transit peptide alone is sufficient to direct a foreign protein into the chloroplast, the mature SSU, or parts thereof, significantly influence transport.

Processing of pSSU has been studied intensively. Incubation of pSSU with partially purified processing enzyme or with chloroplast stroma fractions results in an intermediate. Based on this result, Robinson and Ellis (1984) proposed that maturation of the pSSU occurs in two steps. *Chlamydomonas* pSSU is imported and partially matured by pea and spinach chloroplasts (Mishkind et al. 1985). Cleavage of the *Chlamydomonas* preprotein occurs within a region of the transit peptide which contains the first processing site. This region is conserved among higher plant and algal SSU precursors. Studies in which specific amino acids of pea pSSU were substituted with amino acid analogs have shown that the presence of at least a single proline, arginine and leucine in the precursor protein is essential for both import into chloroplasts and processing by the purified processing enzyme (Robinson and Ellis 1985).

In order to identify which parts of the transit peptide are required for transport, we introduced mutations into the transit peptide and studied the transport of the mutant proteins in vitro. The use of restriction enzymes rather than exonuclease digestion allowed us to create several overlapping deletions and one insertion. The NPTII fusion protein that has the transit peptide attached directly to NPTII was used in order that the function(s) of the transit peptide

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might be studied in the absence of any complex interactions which may exist between the transit peptide and the mature protein. The results show that, although mutants having large deletions in the aminoterminal halves f the transit peptide are transported, their import is severely impaired. Interestingly, some of these mutants lack portions of the conserved region which is reputed to contain the first processing site (Mishkind and Schmidt 1986). In contrast to the decreased import observed for the transit peptide deletion mutants, transport of a fusion protein with a partial duplication of the aminoterminal region of the transit peptide was improved markedly.

Materials and methods

DNA manipulations. DNA manipulations and gel electrophoresis were performed essentially as described by Maniatis et al. (1982). Small scale and large scale isolation of plasmids from *Escherichia coli* was carried out according to Birnboim and Doly as modified by Ish-Horowitz (Maniatis et al. 1982). DNA sequencing was performed according to the method of Maxam and Gilbert (1980).

Plasmid constructions of genes with wild-type transit peptide. To obtain the fusion proteins TPNPTII and TPSSNPTII, the intermediate plasmids described below were used. The gene encoding TPNPTII was constructed by assembling portions of plasmids pTP2, pKM109/15 (Reiss 1982) and ptac12/Hind. Plasmid pTP2 was derived from pTP1 which carries the *Eco*RI-*Sph*I fragment of pPSR6 (Cashmore 1983) that codes for the promoter and transit peptide of SSU in pBR327 (Soberon et al. 1980). pTP1 was linearized by digestion with *Sal*I, the ends of the fragments were filled in using the large fragment of DNA polymerase I and ligated with an equimolar mixture of *Bam*HI (sequence CGGATCCG) and *Sph*I (sequence GGCATGCC) linkers. The ligation products were digested with *Sph*I and the ends of the plasmid were ligated together. The sequence of the resulting polylinker (Fig. 1) was confirmed by DNA sequence analysis. To construct ptac/Hind, plasmid ptac12 (Amann et al. 1983), which contains the hybrid *tac* promoter, was restricted at the unique *Pvu*II site and a *Hin*dIII linker (sequence CAAGCTTG) was attached. pKm109/15 contains the NPTII gene with an upstream *Bam*HI site. To construct pTPK1 carrying the assembled TPNPTII gene, an *Eco*RI-*Bam*HI vector fragment from pKM109/15 was ligated with the *Hin*dIII-*Bam*HI fragment of pTP2 that contains the transit peptide coding sequence and the *Eco*RI-*Hin*dIII fragment of ptac12/Hind that carries the *tac* promoter. The plasmid pSSKM3 which encodes TPSSNPTII consists of a *Sph*I-*Sal*I fragment of pTPK1 as vector, ligated with a *Sph*I-*Sau*3AI fragment which contains the coding sequence of the 23 amino acids from mature SSU from pSS15 (Broglie et al. 1981; Coruzzi et al. 1983) and a *Bam*HI-*Sal*I fragment which contains the NPTII gene from pKM109/9 (Reiss et al. 1984b).

The plasmids ptac/TPNPTII and ptac/TPSSNPTII were derived from pTPK1, pSSKM3, and pOLI. The *Eco*RI-*Eco*RV fragment from pTPK1 or pSSKM3 was exchanged with the analogous fragment from pOLI. The intermediate vector pOLI which introduced an oligonucleotide containing the model ribosome binding site was constructed in the following manner. ptac12 was linearized with *Pvu*II and ligated with two non-phosphorylated complementary synthetic oligonucleotides (sequences 5' CTGATGGCTTCTATGATATC and 5' GATATCATAGAAGCCATCAG, provided by the University of Arizona ligonucleotide synthesis facility) in molar excess. Integration of the oligonucleotides was verified by digestion with *Eco*RI and *Eco*RV and with *Eco*RI and *Pvu*II. To verify the structure of the oligonucleotide junction sequences, the nucleotide sequence of this region of ptac/TPNPTII was determined.

Plasmids pSP64/TPNPTII and pSP64/TPSSNPTII were obtained by inserting the *Hin*dIII-*Sal*I fragments containing the hybrid genes from pTPK1 and pSSKM3, respectively, into the polylinker of pSP64 (Melton et al. 1984). Plasmid pSP64/SSU was assembled from different intermediates and in various steps. The gene construction consists of a *Hin*dIII-*Sma*I vector fragment derived from pSP64/TPSSNPTII, the *Hin*dIII-*Sph*I fragment encoding the transit peptide (derived from pPSR6), the *Sph*I-*Kpn*I fragment encoding the aminoterminal part of the SSU from pSS15, and the *Kpn*I-*Hpa*I fragment carrying the carboxyterminal part of the SSU gene from pPSR6. To obtain authentic NPTII from SP6-transcription and translation in wheat germ extracts, the *Bgl*II-*Sal*I fragment which contains the NPTII gene from pKM2 (Beck et al. 1982) was ligated with pSP65 (Melton et al. 1984) which had been digested with *Bam*HI and *Sal*I.

Construction of plasmids with mutant transit peptides. Restriction endonucleases were used to construct mutants in the coding sequence of the transit peptide in the following manner. A *Bst*XI-*Sal*I fragment which contains the transit peptide coding sequences from pTP2 was isolated and subsequently restricted by one of the following enzymes: *Eco*RV, *Hin*fI, *Nla*IV, or *Hae*III. The ends of the fragments generated by digestion with *Hin*fI were filled in using *E. coli* DNA polymerase large fragment. Mixtures of fragments produced by each enzyme were restricted with *Hin*dIII and *Bam*HI. To produce the various mutants, appropriate restriction digests were mixed and ligated to a *Hin*dIII-*Bam*HI vector fragment derived from pTPK1. The ligation mixtures were used to transform cells of *E. coli* (strain 71-18, Messing et al. 1977). Cells carring recombinant plasmids were selected on ampicillin and subsequently tested for their ability to grow on kanamycin. The correct structure of the mutations was confirmed by digestion with restriction endonucleases. Plasmids ptac/PNi6/25 and ptac/PNd26/35 were derived by cloning *Eco*RV-*Bam*HI fragments containing the mutant transit peptide sequences into a vector fragment produced from ptac/TPNPTII by digestion with *Eco*RV and *Bam*HI. Plasmids ptac/PNd6/25 and ptac/PNd6/29 were derived directly from ptac/TPNPTII by ligating the vector which had been digested with *Eco*RV and *Bam*HI with either a *Nla*IV-*Bam*HI or a *Hae*III-*Bam*HI fragment.

All SP6 vector derivatives were obtained by cloning the *Hin*dIII-*Sal*I fragments that contain the entire mutant gene of the intermediate plasmids into pSP64. DNA sequence analysis was used to verify that the SP6 derivatives of the mutants had the correct structure.

Expression in E. coli. Cultures of *E. coli* (strain 71–18, Messing et al. 1977) harboring the various plasmids were grown overnight in Luria-Bertani (LB) medium containing 100 μg/

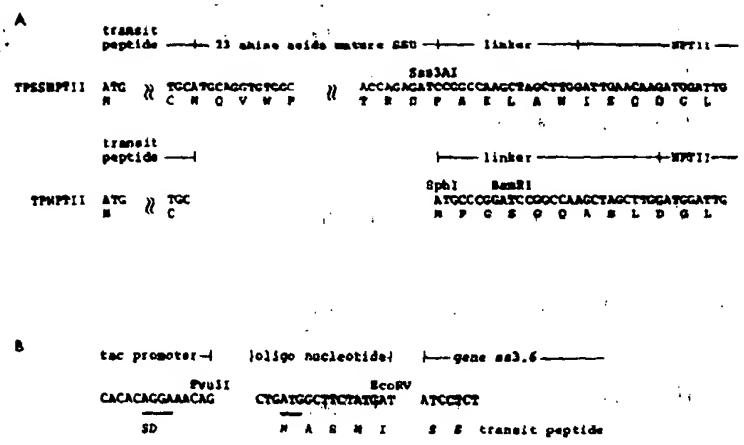

Fig. 1. A Junction sequences of the different gene constructions. B Sequence of the model ribosome binding site used for expression in *Escherichia coli*. The derived amino acid sequence is shown below the DNA sequence. Restriction endonuclease recognition sites pertinent for the gene construction are indicated. The gene fragments or synthetic oligonucleotide sequences from which the chimeric genes were derived are identified. The Shine and Dalgarno (SD) sequence (Shine and Dalgarno 1974) and the initiation codon of the transit peptide are *underlined* in **B**

ml ampicillin. For expression of the cloned genes, the cells were diluted 1/100 in LB medium containing ampicillin and the inducer IPTG (2 mM, isopropyl ß-D-thiogalactoside) and grown to late mid-log phase. The cells were concentrated 20-fold by centrifugation. Crude lysates were prepared by probe sonication in 50 mM Hepes, pH 8, 110 mM K acetate, 0.8 mM Mg acetate. The cell lysates, cleared of cellular debris by centrifugation at 15,000 *g* for 1 min, were used directly for uptake.

Expression in vitro. SP6 transcripts were synthesized and capped essentially as described by Krieg and Melton (1984) with the exception that the DNase treatment was omitted. Plasmid DNA purified on CsCl gradients was linearized with *Sal*I and the templates were transcribed with SP6 polymerase in the presence of RNasin. The RNA transcripts were capped with vaccinia virus guanylyl transferase in the presence of RNasin. The capped synthetic mRNA was translated in wheat germ extracts (typically 30 µl reaction volumes) in the presence of ^{35}S-methionine as specified by the supplier (Bethesda Research Laboratories, Gaithersburg, Maryland). Pea poly(A)$^+$ RNA was obtained as described by Schreier et al. (1985).

In vitro uptake. The in vitro transport experiments, protease treatment, and fractionation were performed essentially as described by Bartlett et al. (1982). Intact chloroplasts were isolated from 10- to 14-day-old pea plants (Ferry Morse, Progress No. 9) using the Percoll gradient technique. Each incubation mixture for uptake contained either 100 µl of *E. coli* cell lysate or 20 µl of wheat germ extract in a standard reaction as described by Bartlett et al. (1982). The mixture was incubated in the light for 1 h. The chloroplasts were recovered by centrifugation, washed twice, and resuspended in a total volume of 80 µl. Treatment of the chloroplasts with exogenous protease was performed in the following manner. The chloroplasts (40 µl) were incubated with trypsin and chymotrypsin (312 µg/ml each) for 15 min in ice water. The protease digestion was terminated as described.

Protein gels and enzymatic assay. NPTII assays were performed as described by Reiss et al. (1984a). SDS-polyacrylamide gel electrophoresis (15% gels) was performed as described by Laemmli (1970) and fluorography was performed as described by Chamberlain (1979).

Results

Constructions with wild-type transit peptide

Two basic fusion proteins were used in these experiments (Fig. 1A). The first fusion protein, TPNPTII, has the transit peptide sequence from the pea SSU gene ss3.6 (Cashmore 1983) connected to the NPTII gene via a short linker. This linker differs from that used by van den Broeck et al. (1985) to obtain an analogous fusion protein. The second fusion protein, described by Schreier et al. (1985), contained the 23 aminoterminal amino acids of mature SSU between the transit peptide and NPTII. However, the gene construction used by Schreier et al. retained the intron located between the codons specifying the second and third amino acids of mature SSU in the gene ss3.6. To obtain our fusion protein, TPSSNPTII, the intron was removed by combining genomic and cDNA (Broglie et al. 1981; Coruzzi et al. 1983) sequences. Similarly, an authentic SSU precursor lacking intervening sequences was obtained by combining cDNA and genomic sequences (see Materials and methods).

Comparison of transport of the fusion proteins TPNPTII and TPSSNPTII by enzymatic activity

TPNPTII and TPSSNPTII were expressed in *E. coli* cells under the control of the inducible *tac* promoter (Amann et al. 1983). A synthetic oligonucleotide was used to provide a model ribosome binding site (Fig. 1B). Precursor proteins and NPTII were obtained as crude lysates of the *E. coli* cells. Authentic NPTII was obtained from cells carrying pKM2 (Beck et al. 1982).

Transport experiments were performed by incubating isolated intact chloroplasts with the preproteins (Bartlett et al. 1982). After the incubation period, the chloroplasts were recovered and the extracts assayed for NPTII activity. For this purpose, the proteins were separated on a nondenaturing polyacrylamide gel and NPTII enzymatic activity was detected by phosphorylation of kanamycin in situ (Reiss et al. 1984a). Radioactive kanamycin phosphate was visualized by autoradiography (Fig. 2). The enzymatic activities obtained with the precursor proteins in the *E. coli* extracts are presented in lanes 1 to 3. The transport experiment is presented in lanes 4 to 9. Whereas TPSSNPTII activity appeared as a single relatively weak spot, TPNPTII showed a spot of high activity that migrated slightly faster

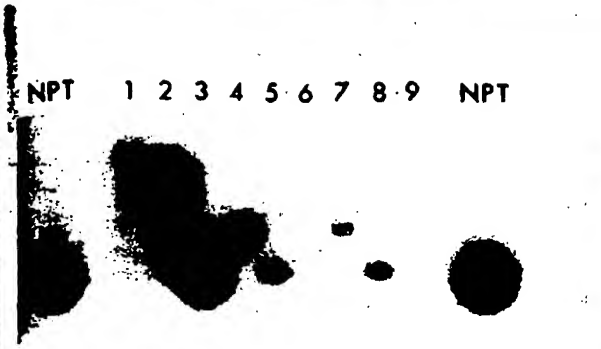

Fig. 2. Protein transport analyzed by assaying neomycin phosphotransferase II (NPTII) activity. Protein extracts were separated on non-denaturing polyacrylamide gels and NPTII enzymatic activity was determined in situ. Extracts of precursor proteins: TPSSNPTII (lane 1), TPNPTII (lane 2), NPTII (lane 3). Chloroplasts before (lanes 4, 5, 6) and after (lanes 7, 8, 9) protease treatment: TPSSNPTII (lanes 4, 7), TPNPTII (lanes 5, 8) and NPTII (lanes 6, 9). NPTII standards (NPT) were derived from pKM2 extracts

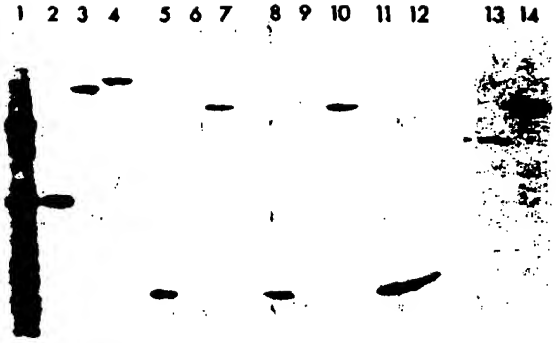

Fig. 3. Transport of labeled precursor proteins. Precursors obtained in vitro from pea poly(A)$^+$ RNA (lane 1), pSP64/SSU (lane 2), pSP64/TPNPTII (lane 3) and pSP64/TPSSNPTII (lane 4). Chloroplasts before (lanes 5, 6, 7, 11) and after (lanes 8, 9, 10, 12, 13, 14) protease treatment: SSU, lanes 5, 8; TPNPTII, lanes 6, 9; TPSSNPTII, lanes 7, 10. Transport of precursor obtained from pea poly (A)$^+$ RNA-primed wheat germ extracts, lanes 11 and 12. Lanes 13 and 14 are identical to lanes 9 and 10, with the exception that a longer exposure is shown

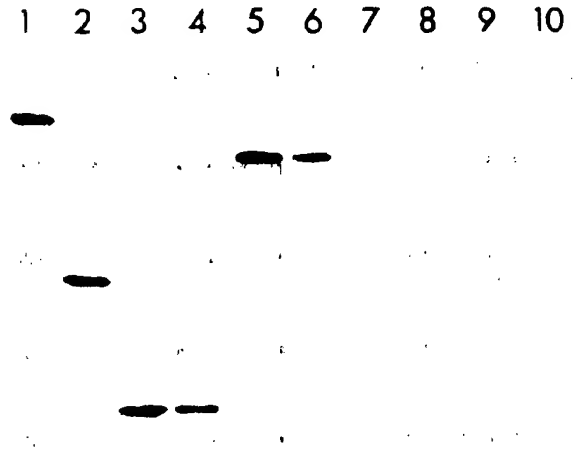

Fig. 4. Fractionation of chloroplasts after uptake of labeled precursors. Precursors: TPSSNPTII (lane 1) and SSU (lane 2). Stroma fractions of chloroplasts incubated with pSSU: chloroplasts without (lane 3) and with protease treatment (lane 4). Stroma fractions of chloroplasts incubated with TPSSNPTII: chloroplasts before (lane 5) and after (lane 6) protease treatment. Membrane fractions from chloroplasts incubated with SSU precursor: chloroplasts without (lane 7) and with (lane 8) protease treatment. Membrane fractions of chloroplasts incubated with TPSSNPTII: chloroplasts without (lane 9) and with (lane 10) protease treatment

than TPSSNPTII. We assume that the difference in the activities observed reflects differences in the expression of the two fusion proteins in *E. coli*. In addition, two minor spots of activity that migrated faster than the major TPNPTII spot are visible. These minor spots correspond to proteins that were also visible in autoradiograms of extracts of *E. coli* cells labeled with ^{35}S-methionine (data not shown). We conclude that these minor spots are unspecific degradation products of the precursor protein produced in *E. coli*.

Extracts from chloroplasts incubated with TPSSNPTII showed a strong signal at a position intermediate between authentic NPTII and TPSSNPTII. A weaker signal at the position of authentic NPTII was observed in extracts of chloroplasts which had been incubated with TPNPTII. This result shows that both fusion proteins were successfully transported. Authentic NPTII offered to chloroplasts in amounts similar to TPNPTII was not imported.

To confirm transport of TPNPTII and TPSSNPTII, the chloroplasts were treated with protease after uptake (Bartlett et al. 1982). Both proteins were inside the chloroplast, as shown by their resistance to protease digestion. However, some decrease in NPTII activity was observed after the protease treatment. This decrease, which is most obvious in Fig. 2 with TPSSNPTII, was observed only when uptake was analyzed under the non-denaturing conditions necessary for NPTII assays. The reduction in activity results from protease digestion during sample processing under native conditions. Consistently, the effect was more pronounced on TPSSNPTII, possibly because this fusion protein is a suitable target for the protease. Therefore, the results obtained before protease treatment more accurately reflect the transport efficiencies of these proteins. Both TPNPTII and TPSSNPTII were transported into chloroplasts while NPTII, which lacks a transit peptide, was not imported by chloroplasts. However, the two fusion proteins appeared to be transported with drastically different efficiencies. Because the specific activity of NPTII fusion proteins depends on the nature of the aminoterminal extension (Reiss et al. 1984b), the differences in NPTII activity which we observed could reflect the differences in sequence between TPNPTII and TPSSNPTII. To resolve this point, we measured import of radioactively labeled precursor proteins, a method which allows a more accurate quantitation of transport since it does not depend on enzymatic activities.

Comparison of transport of pSSU, TPNPTII and TPSSNPTII using radioactively labeled preprotein

In order to obtain radioactively labeled preproteins, the chimeric genes coding for TPNPTII, TPSSNPTII and pSSU were modified to allow transcription by SP6 polymerase. The modified plasmids pSP64/TPNPTII, pSP64/TPSSNPTII and pSP64/SSU were transcribed in vitro, the synthetic mRNA was capped with guanylyl transferase (Krieg and Melton 1984), and translated in wheat germ

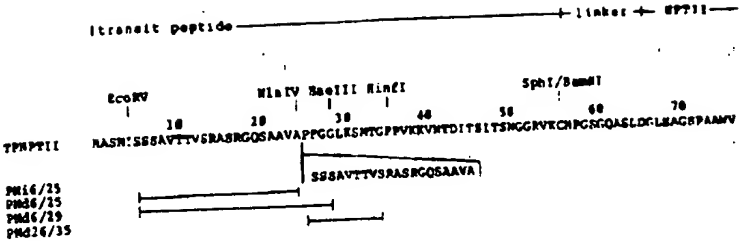

Fig. 5. Schematic representation of mutations in the transit peptide of TPNPTII. The derived amino acid sequence of the transit peptide and the junction between the transit peptide and neomycin phosphotransferase II (NPTII) are shown. The locations of the restriction endonuclease sites referred to in the text are indicated. Deletions are shown as *bars*. The sequence of the partial duplication in the transit peptide is shown

extracts (Bartlett et al. 1982) in the presence of ^{35}S-methionine. Translation of pSP64/SSU RNA yielded a polypeptide identical to that obtained from translation of pea leaf poly(A)$^+$ RNA.

The radioactive precursors were incubated with isolated intact chloroplasts and extracts of chloroplasts analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. The TPSSNPTII protein was transported in amounts comparable to the authentic pSSU (Fig. 3). Import of TPNPTII, which lacks the 23 amino acids from the mature SSU, was detectable only after long exposure times (compare Fig. 3, lanes 9 and 13). Transport of pSSU translated from SP6 RNA was identical to that translated from pea poly(A)$^+$ RNA. NPTII synthesized in vitro was not imported by chloroplasts (data not shown). Resistance of the labeled proteins to externally added protease confirmed that these proteins were inside the chloroplast.

The transported proteins were smaller than the precursors. The apparent molecular weights observed for the mature forms of pSSU and TPNPTII were as expected for the correctly processed proteins. As molecular weight markers NPTII made in bacteria, and the precursor and processed forms of SSU synthesized from pea poly(A)$^+$ RNA were used (data not included). However, the product of processed TPSSNPTII migrated with an apparent molecular weight which was higher than would be expected if 23 amino acids were added to NPTII. Although a gel artifact cannot be excluded, this aberrant migration seems to reflect incorrect processing. The main argument for incorrect processing at present is that only the processed TPSSNPTII behaves abnormally. Concerned that this abnormality might indicate failure of this protein to reach its expected destination, the chloroplast stroma, we separated post-uptake chloroplasts into membranes and stroma. The processed form of TPSSNPTII was found only in the stroma fraction (Fig. 4). From this result we conclude that the TPSSNPTII cleavage product reaches the destination expected of a protein with an SSU transit peptide. Occasionally, additional polypeptides which migrated faster than the TPSSNPTII cleavage product were observed in chloroplasts incubated with TPSSNPTII (see for example Fig. 7B, lanes 6 and 9). Because the appearance of these proteins was independent of treatment with external protease, we conclude that these proteins are either unspecific degradation products of the imported protein or processing intermediates.

In order to exclude the possibility that the inefficient transport of TPNPTII was due to degradation of the preprotein in the uptake mixture, we examined the incubation medium after removal of the chloroplasts. Whereas very little pSSU or TPSSNPTII remained in the medium, TPNPTII was present at nearly its initial concentration (data not shown). We found no evidence that degradation of the precursor occurred during the incubation with the chloroplasts.

Based on data obtained from several experiments we estimated the uptake efficiencies of the various preproteins. Approximately 50% of the offered pSSU and TPSSNPTII molecules were routinely recovered with the chloroplasts. However, based on the small amount of precursor remaining in the incubation mixture after transport, the actual transport efficiencies may be higher. The difference between the two estimates is probably due to the loss of chloroplasts during the treatment after the uptake. In contrast to the efficient import of TPSSNPTII, the transport of TPNPTII was very inefficient. We estimate that the transport efficiency of TPNPTII was 10%, or less, of that of pSSU or TPSSNPTII.

Construction of mutants in the transit peptide of TPNPTII

Mutants in the transit peptide were constructed using restriction endonuclease sites within the coding region of the transit peptide of TPNPTII. Three deletion mutants and one insertion mutant were obtained by combining restriction fragments to create changes within the transit peptide. The derived amino acid sequences of these mutants together with a map of the restriction endonuclease sites used in their construction are shown in Fig. 5.

Analysis of transit peptide mutants using enzymatic activity

Precursor protein was obtained from *E. coli* cells transformed with plasmids, ptac/PNd6/25, ptac/PNd6/29, ptac/PNd26/35, or ptac/PNi6/25, which have the mutant genes under control of an expression unit consisting of the *tac* promoter and the oligonucleotide ribosome binding site described earlier. Mutant precursor proteins were incubated with isolated intact chloroplasts and transport was monitored by assaying NPTII activity (Fig. 6). The NPTII activity migrating at the position of the precursor protein was sensitive to treatment with externally added protease and is probably precursor bound to the outer surface of the chloroplasts. The second activity migrated indistinguishably from authentic NPTII, the product expected for the imported and processed form of TPNPTII. This faster-migrating NPTII-like activity was resistant to protease and thus represents protein residing within the chloroplast. Taking the NPTII-like activities as a measure of transport, uptake of the insertion mutant PNi6/26 appears comparable to that of the wild-type preprotein, TPNPTII. However, shorter exposures than those presented reveal that this insertion actually improves transport. Compared to TPNPTII (Fig. 2), the transport of the deletion mutants PNd6/29 and PNd26/35 was reduced drastically. Uptake of mutant PNd6/25 was not detectable.

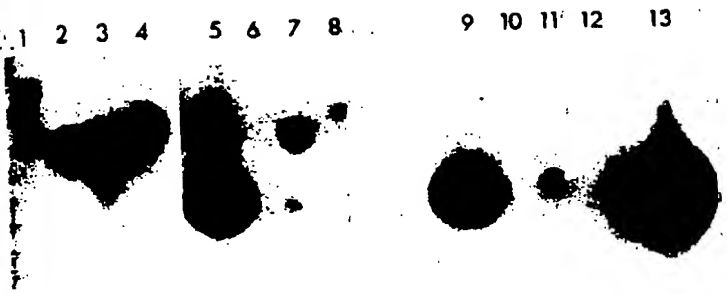

Fig. 6. Transport of precursors with mutant transit peptides. Proteins present in extracts of chloroplasts and cells of *Escherichia coli* were separated on non-denaturing polyacrylamide gels and neomycin phosphotransferase II (NPTII) enzymatic activity was determined in situ. The activities of the precursors are shown in lanes 1 (PNi6/25), 2 (PNd6/25), 3 (PNd6/29) and 4 (PNd26/35). Chloroplasts before (lanes 5, 6, 7, 8) and after (lanes 9, 10, 11, 12) protease treatment: PNi6/25 (lanes 5, 9), PNd6/25 (lanes 6, 10), PNd6/29 (lanes 7, 11) and PNd26/35 (lanes 8, 12). An NPTII standard derived from pKM2 is shown in lane 13

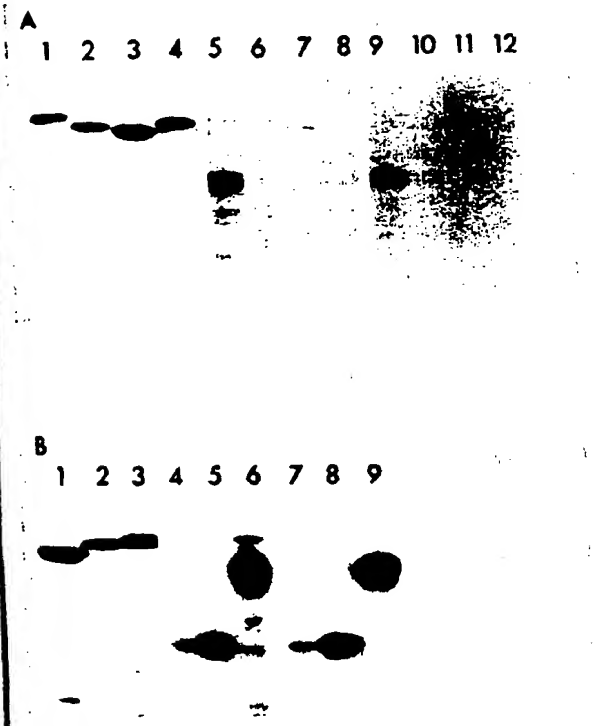

Fig. 7A, B. Transport of radioactively labeled precursors with mutant transit peptides. A Transport of deletion mutants. Precursors: TPNPTII (lane 1), PNd6/25 (lane 2), PNd6/29 (lane 3) and PNd26/35 (lane 4). Chloroplasts before (lanes 5–8) and after (lanes 9–12) protease treatment: TPNPTII (lanes 5, 9), PNd6/25 (lanes 6, 10), PNd6/29 (lanes 7, 11) and PNd26/35 (lanes 8, 12). B Comparison of the transport of the insertion mutants, TPNPTII and TPSSNPTII. Precursors: TPNPTII (lane 1), PNi26/35 (lane 2) and TPSSNPTII (lane 3). Chloroplasts before (lanes 4–6) and after (lanes 7–9) protease treatment: TPNPTII (lanes 4, 7), PNi6/25 (lanes 5, 8) and TPSSNPTII (lanes 6, 9)

Analysis of transit peptide deletion mutants using labeled preprotein

In order to examine uptake of the mutants in a more quantitative manner, the mutants were recloned into SP6 vectors (Melton et al. 1984) to yield plasmids pSP64/PNd6/25, pSP64/PNd6/29, pSP64/PNd26/35 and pSP64/PNi6/25. Radioactively labeled preprotein was synthesized in vitro and incubated with isolated intact chloroplasts. In contrast to the results obtained with the NPTII assay, transport of labeled precursor proteins with truncated transit peptides was not detectable (Fig. 7A). However, the enhanced import of the precursor protein containing the insertion (mutant PNi6/25) was clearly evident (Fig. 7B). Transport of PNi6/25 was considerably more efficient than that of TPNPTII, but less efficient than that of TPSSNPTII. In contrast to TPSSNPTII, the PNi6/25 preprotein appeared to be processed correctly upon import into chloroplasts.

Discussion

We compared the transport by intact chloroplasts of two transit peptide-NPTII fusion proteins which differed by 23 amino acids of mature SSU sequence. Two methods for detecting the proteins in chloroplast extracts were used to determine the relative transport efficiencies of these proteins. The enzymatic activity of the NPTII fusion proteins synthesized in *E. coli* was assayed to provide a sensitive method for comparison of TPNPTII and TPSSNPTII. This method had been used in previous studies (Van den Broeck et al. 1985; Schreier et al. 1985) and thus allowed a comparison of our results with those obtained previously. In order to obtain more quantitative data on the import of the TPNPTII and TPSSNPTII proteins, we used radioactively labeled precursors synthesized in vitro. This method also enabled us to compare the import of the NPTII fusion proteins with that of authentic pSSU. The results obtained with both methods agreed and showed that, although the transit peptide alone was sufficient to direct NPTII into chloroplasts, transport was strongly influenced by the additional 23 amino acids present in the TPSSNPTII protein. Protein TPSSNPTII was imported with an efficiency comparable to that of authentic pSSU while the TPNPTII polypeptide was imported with a relatively low efficiency.

To examine the transit peptide in more detail, mutations were introduced into the transit peptide of TPNPTII. All of the deletion mutations examined were located in the aminoterminal half of the transit peptide. Two of the mutants (PNd6/25 and PNd6/29) have overlapping deletions. One of these, PNd6/29, and deletion mutant PNd26/35 were imported into chloroplasts with drastically reduced efficiencies. Transport of mutant PNd6/25 was not detectable. An insertion mutation, containing a partial duplication of the transit peptide, increased the transport of the TPNPTII fusion protein.

The question arises as to whether specific sequences in the mature part of the SSU precursor are necessary to obtain optimal transport or if other factors are involved. Considering that the majority of the proteins found inside the

chloroplast are imported from the cytoplasm, the presence of a common specific sequence required for transport into the stroma appears unlikely. The amino acid sequences of the precursors of two stromal proteins, ferredoxin (Smeekens et al. 1985) and SSU (Cashmore 1983), are available. Surprisingly, the ferredoxin transit peptide shares homology with a portion of the first 23 amino acids of mature SSU. The homologous residues in ferredoxin are the three charged residues K-Q-Q and the sequence S-S-L-P. These amino acids correspond to the sequence K-K-K and S-Y-L-P in the mature SSU. Whether this homology is fortuitous or whether it influences the targeting of a protein towards different chloroplast compartments remains to be studied. In view of this homology, the requirement of a specific sequence within the 23 amino acids cannot be excluded. However, it appears more probable that other information contained in the 23 amino acids, e.g., charge distribution, is necessary for transport. Alternatively, the three-dimensional structures of the precursors may be important. It is conceivable that the precursor molecule must assume a particular conformation in order to be recognized by the putative receptor complex in the chloroplast envelope. Two different conformations for the transit peptide-NPTII fusion protein can be envisioned. Either the transit peptide and parts of NPTII form a single domain or the transit peptide and the NPTII portions form separate domains. In a single domain model, the correct folding of the NPTII portion might impose an aberrant conformation on the transit peptide. This could interfere with its recognition by the receptor complex. In a two domain model, the transit peptide part might be concealed by the NPTII domain leading to inefficient interaction between the precursor and the receptor. In contrast to the TPNPTII protein, the transit peptide of TPSSNPTII is separated from NPTII by an additional 23 amino acids. Possibly, this separation of the transit peptide from the NPTII moiety allows the transit peptide to assume its native conformation or renders it more accessible to the receptor. The increase in transport observed with the insertion mutant is consistent with the hypothesis that a conformational distortion leads to inefficient recognition by the receptor. The effect of the insertion in the transit peptide may be similar to the effect of the 23 amino acids of mature SSU. That is, the insertion results in an altered conformation which improves recognition by the receptor.

The imported form of the TPSSNPTII polypeptide had an apparent molecular weight that appears larger than expected. It is possible that in this protein some part of the transit peptide which influences maturation is folded incorrectly and cannot be recognized by the processing enzyme. Robinson and Ellis (1984) have shown that processing of pSSU proceeds via an intermediate in vitro. The cleavage that yields the mature SSU is not required for transport into the stroma (Robinson and Ellis 1984). Thus, incorrect processing at this point would not be expected to interfere with transport. We assume that the maturation cleavage of the TPSSNPTII polypeptide is inhibited. Therefore, the imported protein could be the product of the first processing step. The incorrect processing of the TPSSNPTII protein appears to be inconsistent with the hypothesis that a conformational distortion interferes with transport. However, as transport and maturation are independent processes (Robinson and Ellis 1984), they may be affected differently by a particular conformation.

Our deletion mutations are in the region of the transit peptide containing the putative first processing site and, therefore, at least one of these mutants lacks this site. Two of these mutants, PNd6/29 and PNd26/35, are transported into the chloroplast, although with greatly reduced efficiencies. Perhaps, the sequence surrounding the first processing site is not essential for the first cleavage or, alternatively, the first cleavage is not a required step in transport. The processing enzyme may recognize sequences outside of the deleted region. Alternatively, the processing enzyme may measure from the aminoterminus of the transit peptide. Robinson and Ellis (1984) invoked a similar hypothesis to explain the maturation of a modified precursor.

Protein transport into mitochondria has been studied in detail (Hurt et al. 1984, 1985; Schatz and Butow 1983; Horwich et al. 1985). The transit peptide of subunit IV of yeast cytochrome oxidase fused to the aminoterminus of mouse dihydrofolate reductase directs the enzyme into the matrix of isolated mitochondria (Hurt et al. 1984). Further analysis of deletions of the presequence showed that the 12 aminoterminal residues of the transit peptide are sufficient for transport. In this paper we show that precursors which lack portions of the aminoterminal region of the transit peptide from the SSU are transported with drastically reduced efficiency. Although transport is not abolished by all of the mutations, the aminoterminal region of the transit peptide appears to be important for function. From our results we conclude that with respect to the requirement of the aminotermini of the transit peptides the mitochondrial and chloroplast transport systems are similar.

Transport of only two, PNd6/29 and PNd26/35, of the three truncated polypeptides could be detected. Interestingly, the sequence that is deleted in the third mutant, PNd6/25, is completely overlapped by the other two mutants. It cannot be determined from our results whether the deletion in PNd6/25 removed necessary sequence information which was restored in mutant PNd6/29 by upstream sequences or if these effects result from different conformations of the transit peptide. The insertion mutant PNi6/25 has an exact duplication of the region of the transit peptide that was deleted in mutant PNd6/25. Transport of the PNi6/25 polypeptide was nearly as efficient as transport of the TPSSNPTII polypeptide. Therefore we conclude that the information encoded in the transit peptide can be separated without interfering with its function. Insertion mutants of this type might be useful for improving transport of foreign proteins into the chloroplast.

In summary, our results show that the pSSU transit peptide can direct transport of a foreign protein, NPTII, into the chloroplast stroma. However, we also suggest from our results that the mature SSU may play an important role in transport. Based on the results obtained with mutations in the transit peptide, we suggest that the aminoterminal region of the transit peptide is important for function.

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